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Ectopic recombination in the central and peripheral nervous system by *aP2/FABP4-Cre* mice: Implications for metabolism research

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ABSTRACT

***aP2-Cre* mice have amply been used to generate conditional adipose selective inactivation of important signaling molecules. We show that the efficiency of Cre mediated recombination in adipocytes and adipose selectivity is not always guaranteed. In particular, Cre activity was found in ganglia of the peripheral nervous system (PNS), in adrenal medulla and in neurons throughout the central nervous system (CNS). Because these tissues have an important impact on adipose tissue, care should be taken when using *aP2-Cre* mice to define the role of the targeted genes in adipose tissue function.**

1. Introduction

Mouse knockout studies have importantly contributed to novel insights in the role of signaling molecules that impact on adipocyte differentiation and function. In some instances conditional cell type selective knockouts had to be generated to circumvent embryonic lethality of global mouse knockouts [1–3]. Adipocyte selective inactivation of genes was possible through the identification of promoter sequences of the *aP2* (fatty acid binding protein 4, *FABP4*) gene [4] and the introduction of Cre/loxP technology [5]. In recent years, these adipocyte restricted knockouts were also used with the particular purpose to define the function of a protein in these lipid storing cells, independent of its role in other tissues [6–8], or to uncover the function of the protein in mature adipocytes as opposed to its role during adipogenesis [9].

Two different mouse lines have been generated expressing Cre recombinase driven by the 5.4 kb promoter/enhancer of the *aP2/FABP4* gene. The *aP2-Cre* mice generated by Kahn et al. were reported to exhibit variable and mosaic Cre activity in white (WAT) and brown adipose tissue (BAT) (70–99%) [6,8]. In the other *aP2-Cre* line created by Evans et al. (available from Jackson Laboratory) [1], Cre is also expressed in both adipose tissues, but, surprisingly, it was recently shown that Cre is ectopically active in developing trigeminal ganglia, dorsal root ganglia, cartilage primordia and vertebrae [10]. Because the latter osteoblastic tissues were shown to contain increased numbers of lipid droplets and since they share a mesenchymal stem cell progenitor in common with adipocytes, it was concluded that these cells have adipocyte-like properties [10]. Our studies now reveal that the *aP2* promoter also drives gene inactivation in different brain regions, including the hypothalamus, which is a central notch in regulating metabolism homeostasis [11,12], as well as in the peripheral nervous system (PNS) which is an essential player in the control of adipose tissue function [13]. In our investigations, the two published *aP2-Cre* lines were used, which were bred with either *lacZ* reporter mice [14] or with floxed *Pex5* mice [15].

2. Materials and methods

2.1. Chemicals

Enzymes were purchased from Roche (Mannheim, Germany), chemicals from Sigma–Aldrich (Bornem, Belgium), primers and

Abbreviations: BAT, brown adipose tissue; CNS, central nervous system; FABP, fatty acid binding protein; GFAP, glial acidic fibrillary protein; β-gal, β-galactosidase; NeuN, neuronal nuclei; PBS, phosphate buffered saline; PNS, peripheral nervous system; WAT, white adipose tissue

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probes from Eurogentec (Seraing, Belgium) and organic solvents from Biosolve (Valkenswaard, the Netherlands) or Acros (Geel, Belgium).

2.2. Mouse breeding

Two different *aP2-Cre* mouse lines were used. The *aP2-Cre1* line (strain B6.Cg-Tg(Fabp4-Cre) 1Rev/J) was obtained from The Jackson Laboratory (Bar Harbor, ME) [1] and the *aP2-Cre2* line was kindly provided by Kahn (Boston, USA) [6]. The first Cre line was crossed with either the *LacZ-ROSA* (*R26R*) reporter mouse (strain B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor}) from The Jackson Laboratory [14] or with floxed *Pex5* mice (Swiss Webster) [15]. The second Cre line was only crossed with floxed *Pex5* mice [15]. Genotyping was performed as previously reported [16]. Upon expression of Cre in the *LacZ* reporter mouse, a floxed stop codon preceding the *LacZ* gene is excised resulting in expression of β-galactosidase (β-gal). In the *aP2-Pex5* knockout mice, expression of Cre leads to excision of four essential exons of the *Pex5* gene, resulting in loss of import competent peroxisomes. All mice were 4 weeks old at the time of analysis, unless otherwise indicated.

All experiments were performed in accordance with the "Guidelines for Care and Use of Experimental Animals" and fully approved by the Research Ethical committee of the K.U. Leuven.

2.3. Histochemistry and immunohistochemistry

For X-gal staining, anaesthetized mice were perfused intracardially with 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 in phosphate buffered saline (PBS), pH 7.4. After dissection, tissues were post-fixed for 3 hours. The X-gal staining consisted of incubation at 30 °C overnight in the staining solution (1 mg/ml X-gal solution (50 μg/ml in dimethylformamide, Invitrogen, Merelbeke, Belgium), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS, pH 7.4). Whole organs were washed with PBS, refixed overnight in 4% formaldehyde and embedded in paraffin. 5 μm thick sections were counterstained with Nuclear fast red (Solvent blue 38).

For immunofluorescent stainings, mice were perfused with 10% neutral buffered formalin and sectioned into 10 μm frozen sections as previously described [17]. The following primary antibodies were used: glial acidic fibrillary protein (GFAP) (Sigma, Bornem, Belgium) and neuronal nuclei (NeuN) (Chemicon, Temecula, USA) raised in mice at a 1/100 dilution; catalase (Rockland, Gilbertsville, PA) and β-galactosidase (Cappel, CA, USA) raised in rabbits at a 1/100 and 1/400 dilution.

2.4. Northern blot analysis

Total RNA was isolated using the Trizol® reagent (Invitrogen, Merelbeke, Belgium) according to the manufacturer's specifications. Northern blot analysis was performed as previously described [18]. The blots were hybridized with a *Pex5* probe directed to the 5′ region of the transcript and quantified via ImageJ®. The percent recombination is determined as the ratio of radioactivity present in the recombined *Pex5* band to the total radioactivity in wild type and recombined transcripts.

3. Results

3.1. aP2-Cre1 mice cause gene recombination in the CNS and PNS, besides adipose tissue

aP2-Cre1 mice were bred with *LacZ-ROSA (R26R)* reporter mice [14] to visualize the tissues in which Cre mediated recombination occurs. As expected, in 4-week-old mice, β-galactosidase activity was present in WAT (Fig. 1A) and in BAT (Supplementary Fig. 1A), while no expression could be detected in heart, skeletal muscle, pancreas or liver nor in adipose tissue of control mice (Supplementary Fig. 1A). X-gal staining was also observed in dorsal root ganglia and in vertebrae as previously reported [10] but also in celiac and superior cervical ganglia (Fig. 1B–D), in the medulla – but not in the cortex – of the adrenals (Fig. 1E) and in the skin (Supplementary Fig. 1A). Remarkably, recombination was also detected throughout the brain including the cortex, hippocampus, hypothalamus (Fig. 1F, G, and I), cerebellum (Fig. 1H), spinal cord but not in corpus callosum. In order to evaluate at which age this ectopic recombination occurs, X-gal staining was repeated on brain and spinal cord of 1-day-old pups. Similar as in adult mice, blue staining was observed throughout the brain and spinal cord. To assess which cell types in the brain expressed Cre recombinase, double immunofluorescent stainings were performed of β-galactosidase with either NeuN, which detects most neuronal cell types or with GFAP which is used as a marker for astrocytes. This clearly demonstrated that Cre recombinase fully colocalised with neurons but not with astrocytes (Fig. 1G–I).

3.2. aP2-Pex5 mice loose import competent peroxisomes in ganglia and brain besides adipose tissue

Since *LacZ* reporter mice do not allow to judge the functional consequences of Cre recombination, *aP2-Cre1* mice were bred with another floxed mouse line, *in casu Pex5-loxP* mice [15], in which a crucial gene for import of peroxisomal matrix proteins was floxed. Northern blot analysis was performed to determine the expression of the wild type *Pex5* transcript versus the shorter transcript derived from the recombined allele. In six mice from independent litters we found that the *Pex5* gene was excised by 78.3 ± 2.7% in WAT and BAT (Fig. 2A), which is excellent given the presence of nonadipose cells in these tissues. This was confirmed by quantitative RT-PCR on the wild type *Pex5* transcript which was reduced to 16.3 ± 3.5% in the knockout mice. In agreement with previous reports and with the LacZ data no recombination was noticed in liver, skeletal muscle, heart and kidney (Supplementary Fig. 2A). Recombination in brain was 60.1 ± 5.3% ($n = 4$) which is also in line with the data of the *LacZ* reporter mice (Fig. 2A). In contrast to the LacZ experiments, no shortened *Pex5* transcript was found in adrenals (combined medulla and cortex) (Fig. 2A).

To investigate *Pex5* gene recombination at a cellular level, the ability of peroxisomes to import matrix proteins was investigated in peripheral ganglia, adrenal medulla and brain by examining the subcellular distribution of catalase, a peroxisomal marker enzyme. Whereas in control tissue the typical peroxisomal punctuate pattern of catalase was detected, an estimated 50% of cells in the hypothalamus and in dorsal root ganglia showed a cytosolic location of catalase (Fig. 2B). In adrenal medulla chromaffin cells, only the regular punctuate staining of catalase was observed, in agreement with the northern blot data (Fig. 2B). These results demonstrate that the *aP2* promoter driving Cre expression causes inactivation of peroxisomes in the central and peripheral nervous system.

3.3. aP2-Cre2 mice cause variable recombination in adipose tissue

The efficiency and selectivity of Cre recombinase activity elicited by *aP2-Cre2* mice was examined by breeding with *Pex5-loxP* mice and performing northern blot analysis. In gonadal and subcutaneous WAT recombination of the *Pex5* gene were, respectively, 22.2 ± 0.4% and 48.9 ± 2.1%, whereas in BAT 47.4 ± 1.9% of *Pex5* transcripts were present in the recombined band. In view of this very low recombination efficiency in the tissues of interest, this mouse line was not useful for further research and other tissues

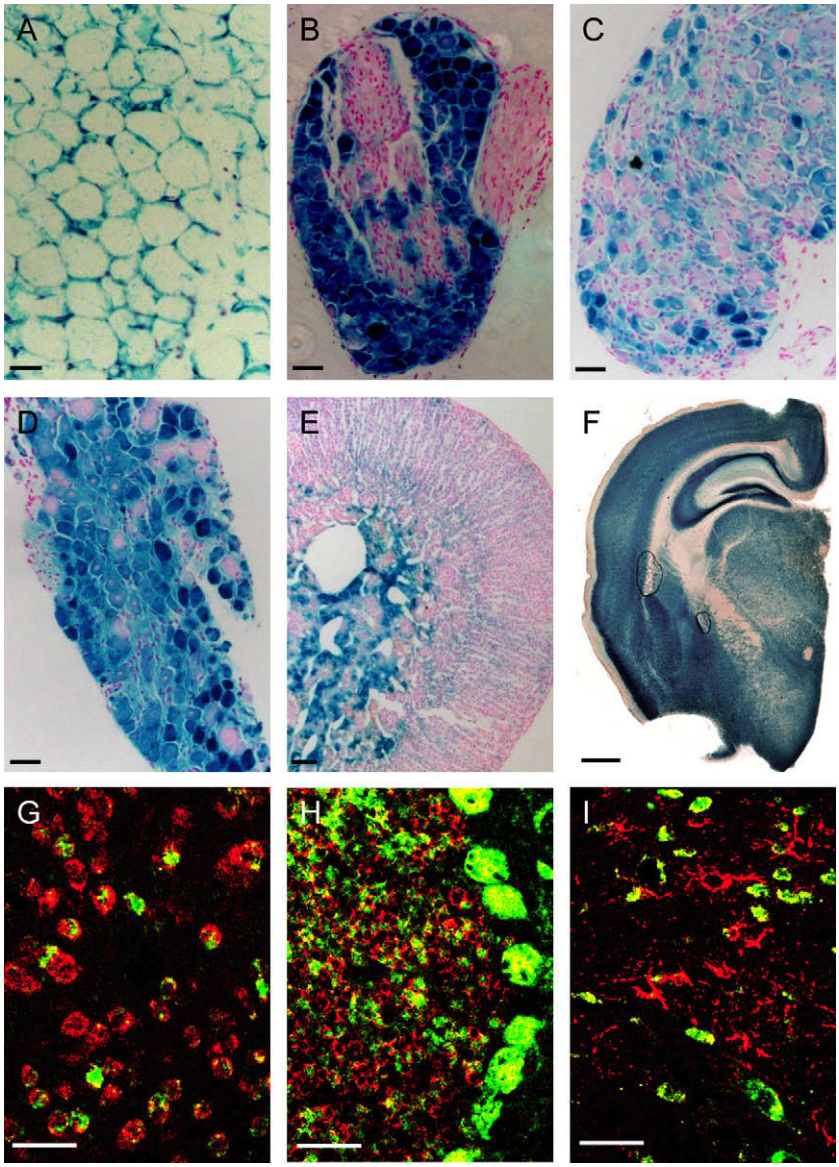

Fig. 1. Localisation of β-galactosidase reporter gene recombination induced by *aP2-Cre1* mice. To visualize β-galactosidase expression either X-gal staining (A–F) or β-galactosidase immunohistochemistry (G–I) was used. In 4-week-old *aP2-Cre1-LacZ*$^{Fl/Fl}$ mice β-gal activity was present in white adipocytes (A), in neural crest derived tissues such as dorsal root ganglia (B), celiac (C) and superior cervical ganglia (D), in the adrenal medulla (E) – but not in the cortex of this tissue – and in brain (F, coronal section). In brain, β-galactosidase expression (green) colocalised with a neuronal marker (NeuN, red) in cortex (G) and cerebellum (H) but not with an astrocytic marker (GFAP, (I) red). Scale bar: (A–D), 25 μm; (E), 50 μm; (F), 1000 μm; and (G–I), 20 μm.

were not examined. Indeed, a recombination efficiency below 50% does not permit to study the consequences of gene ablation in a particular tissue.

4. Discussion

Over the last decade, numerous genetic studies have relied on the use of Cre-driver lines to draw conclusions about the role of signaling molecules in metabolic tissues of mice. In many cases, entirely novel concepts were deduced from such studies, haralding innovative research avenues. However, sometimes, expression of the *Cre* transgene has been more promiscuous than originally anticipated. This is not necessarily problematic, at least as long as such ectopic Cre expression is recognized.

The 5.4 kb promoter/enhancer of the *aP2/FABP4* gene which is generally thought to provoke adipose specific expression appears to be prone to diverse problems of efficiency, consistency and selectivity. The *aP2-Cre2* line, in particular, displayed variable efficiency of recombination in WAT and BAT as already reported [6,8]. In fact, we observed much lower efficiencies in all the mice that we tested preventing to use them for functional studies of the protein of interest. Possibly, this low Cre activity is due to the particular breeding circumstances or genetic background.

With regard to selectivity, expression of Cre driven by the *aP2/FABP4* promoter occurs predominantly in WAT and BAT and in bone marrow, which all contain adipocytes. However, aP2-Cre expression was recently also reported in macrophages and endothelial cells [19–22]. The efficiency of Cre in the latter cell types was lower than in adipocytes based on non quantitative RT-PCR analysis [21]. Ectopic activity of the *aP2* promoter was also recognized in certain progenitor cells during embryonic development by Urs et al. [10]. In the present study, we found besides recombination in osteogenic cells and dorsal root ganglia, additional Cre activity in other ganglia of the peripheral nervous system such as

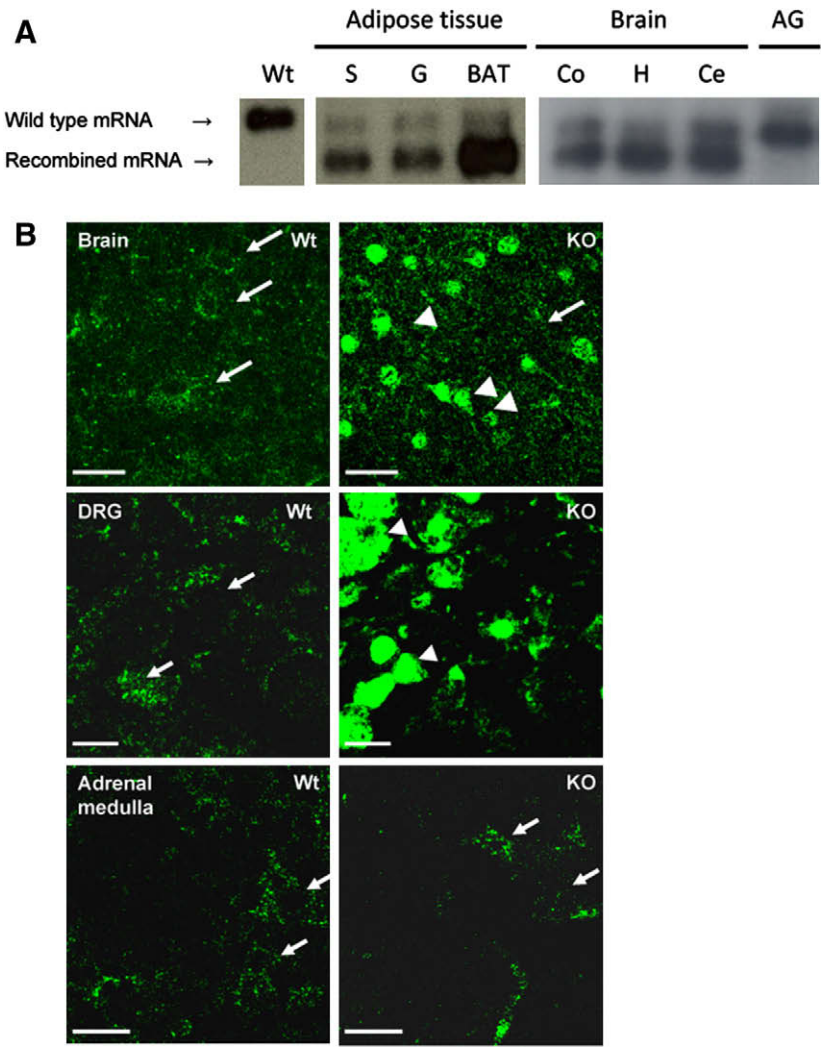

Fig. 2. *aP2-Cre1* mice drive *Pex5-loxP* gene recombination in adipocytes, in brain and in ganglia. (A) Northern blot on tissues of *aP2-Pex5* knockout mice shows the relative abundance of the full length wild type *Pex5* mRNA and the shorter transcript derived from the recombined *Pex5* allel. In subcutaneous (S) and gonadal (G) WAT and in BAT the majority of the *Pex5* alleles are recombined, as expected. Notably, in different brain areas (cortex (Co), hypothalamus (H) and cerebellum (Ce)) more than 50% of transcripts are from the recombined allele, however, no short *Pex5* fragment could be detected in adrenal gland (AG). (B) Sections of the brain, of dorsal root ganglia (DRG) and of adrenals were stained with antibodies to the peroxisomal marker enzyme, catalase showing a punctuate peroxisomal pattern in wild type mice (arrows) and a mixed cytosolic (arrowheads) and peroxisomal pattern (arrows) in brain and DRG of *aP2-Pex5* knockout mice. In adrenal medulla, a normal peroxisomal pattern was observed in the knockout mice. Scale bars: brain, 20 μm; DRG, 10 μm, and adrenal, 10 μm.

celiac and superior cervical ganglia, also in skin and in adrenal medulla. This particular tissue distribution strongly suggests that Cre was expressed in trunk neural crest cells in early embryonic development. Furthermore, strong Cre activity was noticed in the prenatal CNS. In a few previous studies, it was explicitly mentioned that *aP2-Cre* was not active in brain [9,23], but in most other reports no thorough evaluation of the target gene recombination in all relevant tissues was performed. The activity of Cre in adrenal medulla was contradictory between the *LacZ* reporter mice, showing blue staining, and the *aP2-Pex5* knockout mice, which contained normal import competent peroxisomes. A likely explanation is that X-gal staining already appears when a single floxed *lacZ* allele is recombined, whereas both floxed *Pex5* alleles need to be inactivated in order to impair peroxisomal import, possibly requiring higher Cre expression. This underscores that analysis of cell type selective gene recombination, should preferentially be performed with the gene of interest.

In their study [10], Urs et al. come to the conclusion that the embryonic expression of Cre is only relevant when targeting genes that are expressed during embryonic development. In adulthood, expression of endogenous *aP2* and of *aP2* driven constructs might indeed become more adipose selective. However, because Cre mediated recombination is irreversible, all tissues that derive from the progenitor cells in which recombination occurred, will have impaired function of the targeted gene.

A possible strategy to avoid the problem of embryonic expression of Cre, is to use a transgenic mouse line that expresses the tamoxifen inducible fusion protein of Cre recombinase with a mutated ligand binding domain of the human estrogen receptor under the control of the *aP2* promoter [24]. Only after treatment of the mice with tamoxifen, Cre mediated recombination will occur. An alternative is the use of another adipose specific promoter, such as the adiponectin promoter [25]. Also in this case, caution is necessary because new studies revealed that adiponectin is also secreted by other cell types, such as hepatocytes, myotubes, skeletal muscle and osteoblasts [26].

Considering the strong endocrine interactions between adipose tissue, liver, muscle and pancreas, it is certainly important that no Cre mediated inactivation of the target gene occurred in these tissues, as also concluded in previous reports. However, in view of the

extensive innervation and adrenergic control of both WAT and BAT, the widespread *aP2-Cre* driven recombination in the CNS and PNS is highly relevant and urges for caution when one strives to investigate adipocyte autonomous effects of gene inactivation [12,13,27].

In conclusion, depending on the objectives of the research, potential ectopic expression should be taken into account when using *aP2-Cre* mice to investigate the role of candidate regulators of adipocyte function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.01.061.

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